Rab6 and the secretory pathway affect oocyte polarity in *Drosophila*

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The *Drosophila* oocyte is a highly polarized cell. Secretion occurs towards restricted neighboring cells and asymmetric transport controls the localization of several mRNAs to distinct cortical compartments. Here, we describe a role for the *Drosophila* ortholog of the Rab6 GTPase, Drab6, in establishing cell polarity during oogenesis. We found that Drab6 localizes to Golgi and Golgi-derived membranes and interacts with BicD. We also provide evidence that Drab6 and BicD function together to ensure the correct delivery of secretory pathway components, such as the TGFβ homolog Gurken, to the plasma membrane. Moreover, in the absence of Drab6, osk mRNA localization and the organization of microtubule plus-ends at the posterior of the oocyte were both severely affected. Our results point to a possible connection between Rab protein-mediated secretion, organization of the cytoskeleton and mRNA transport.

KEY WORDS: Rab6, Secretory pathway, RNA transport, Gurken, Microtubules, Bicaudal D, oskar mRNA

INTRODUCTION

By regulating the transport of proteins and lipids toward the plasma membrane, the secretory pathway plays an important function in cell polarity. Rab GTPases are important regulatory factors of vesicular traffic. Members of the Rab6 family regulate protein transport between the Golgi, endoplasmic reticulum, plasma membrane and endosome (Del Nery et al., 2006; Mallard et al., 2002; Martinez et al., 1997; Martinez et al., 1994; Opdam et al., 2000). The role of Rab6 in establishing cell polarity was, however, unclear. We chose the *Drosophila* oocyte as a model to study cell polarity in vivo. The oocyte lies at the posterior of the egg chamber, which consists of a cluster of 16 interconnected germ cells surrounded by a monolayer of follicular epithelium. During egg chamber formation, the germ line forms a 16-cell cluster and in which one cell is singled out to become the oocyte, while its 15 sister cells develop into nurse cells (for a review, see Huynh and St Johnston, 2004). During early oogenesis, microtubules (MTs) are nucleated from the MT-organizing center at the posterior of the oocyte. Towards stage 7, an unidentified signal from the posterior follicle cells triggers the organization of perpendicular MT subsets controlling the dorsoventral axis (DV) and anteroposterior axis (AP) in the oocyte (Januschke et al., 2006; MacDougall et al., 2003). bicoid, oskar (osk) and gurken (grk) mRNAs, which determine the embryonic axes, are then localized, respectively, to the anterior, posterior and anterodorsal poles of the oocyte (Riechmann and Ephrussi, 2001).

Studies in cultured mammalian cells have revealed a molecular mechanism whereby BicD modulates MT-based Golgi trafficking by recruiting cytoplasmic Dynein to transport vesicles (Hoogenraad et al., 2001; Matanis et al., 2002). The recruitment of Dynein to vesicles is mediated by the interaction of BicD with the small GTP-localized GTPase Rab6 (Matanis et al., 2002; Short et al., 2005). In *Drosophila*, and more specifically in the oocyte, the Golgi apparatus is not organized into stacked cisternae arranged into ‘Golgi ribbons’. Instead, it is organized into mini-stacks of transitional endoplasmic reticulum (tER)-Golgi units evenly distributed throughout the cell (Herpers and Rabouille, 2004; Kondylis et al., 2001). Whereas three Rab6 isoforms have been characterized in mammals (Del Nery et al., 2006; Mallard et al., 2002; Martinez et al., 1997; Martinez et al., 1994; Opdam et al., 2000), only one has been identified in *Drosophila* (Shetty et al., 1998). So far, *Drosophila* Rab6 (Drab6) has been shown to be involved in Rhodopsin transport in photoreceptor cells and bristle morphogenesis (Purcell and Artavanis-Tsakonas, 1999; Shetty et al., 1998). In this study, we present the characterization of the function of Drab6 during oogenesis and propose a possible connection between Rab protein-mediated secretion, the organization of the cytoskeleton and mRNA transport.

MATERIALS AND METHODS

Fly stocks

*w¹¹¹D* used as wild type; *rab6¹²¹D* (Purcell and Artavanis-Tsakonas, 1999) was recombined to FRT-40A (Bloomington). *rab6¹²¹D* and *khc¹×⁰⁸⁸* germ line and follicle cells clones were generated as described previously (Januschke et al., 2002). GFP-trap, GalT and PDI (A. Debec, Institut Jacques Monod, Paris, France). *khc¹×⁰⁸⁸* (L. Clark, UCLA, Los Angeles, CA), *BicD-GFP* and *BicD-GFP* vectors. Details of protocols for biochemistry experiments are available upon request. The yeast two-hybrid screen was carried out as described (Formstecher et al., 2005).

Electron microscopy and immunodetection were performed as described (Januschke et al., 2006). Antibodies: Stau (St Johnston et al., 1991); Osk (Hachet and Ephrussi, 2001); β-galactosidase (Roche); Grk (DSHB); Lva (W. Sullivan, University of California, Santa Cruz, CA); BiC-D, Syntaxin 5 (DSHB); KDEL (Stressgen); Dynactin (E. L. Holzbaur, Rensselaer Polytechnic, Troy, NY); GFP (Roche); LE lectin (Vector); WGA, phalloidin, LysoTracker (Molecular Probes).

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RESULTS AND DISCUSSION

In vertebrate cells, Rab6 is associated with the Golgi and the trans-Golgi network (TGN) membranes (Del Nery et al., 2006; Mallard et al., 2002; Martinez et al., 1997; Martinez et al., 1994; Opdam et al., 2000). To investigate the subcellular localization of Drab6 in the Drosophila germ line, we monitored the expression pattern of transgenic lines expressing Drab6 fused to GFP (Fig. 1A) and RFP (Fig. 1B). We observed that during oogenesis, the global distribution of Drab6 evolved. Drab6 first accumulated transiently in a central position during stages 7/8, then was uniformly distributed at the beginning of stage 9 to end up juxtaposed to the entire oocyte cortex (Fig. 1A). It is noteworthy that promoters of different strengths gave similar expression patterns. In addition, the genomic null allele rab6^{D23D} (Purcell and Artavanis-Tsakonas, 1999) was fully rescued by the different lines expressing Drab6.

Drab6 did not colocalize extensively with ER membranes (labeled with PDI-GFP) (Bobinnec et al., 2003) (Fig. 1B). Instead, it seemed to be differentially associated with two types of Golgi unit (Fig. 1C). Lava Lamp (Lva), a cis-Golgi marker (Papoulas et al., 2005), colocalized with Drab6, mainly at the cortex of the oocyte and in nurse cells. A GFP trap protein corresponding to a UDP-galactose:beta-N-acetylglucosamine

**Fig. 1. Drosophila Rab6 shows a dynamic localization and is enriched on Golgi membranes.** (A) Drab6 mutant oocytes rescued by GFP-Drab6 expression showed a stage-dependent distribution. Drab6 was central during stages 7 and 8 (arrow, in 40% of cases Drab6 expression was central, n=112), uniform during stage 9 (86%, n=81) and always juxtaposed to the oocyte cortex from the end of stage 9 onward (n=64). (B) RFP-Drab6 and PDI-GFP co-expressing egg chamber. (C) Colocalization of Drab6 and effects of its loss on different Golgi markers in control (I, II, IV, V, VII, VIII) and rab6^{D23D} (III, VI, IX) oocytes. Lva (I) colocalized with Drab6 in a rescued egg chamber mainly at the cortex (II, arrows), but global Lva localization did not depend on Drab6 (III). GalT (IV) colocalized with RFP-Drab6 in the center during stage 8 (V, arrow; inset in IV is a stage 10 egg chamber), but did not accumulate in the center in rab6^{D23D} (VI). WGA was central during stage 8 (VII arrow), colocalized with GalT (VIII, arrow), but formed abnormal ring-like aggregates in rab6^{D23D} (IX, arrow in inset, which is a magnified view of the boxed area). (D) Immunoblots of fractions from a membrane density gradient of GalT-expressing ovaries tested with markers specific to the Golgi (Dynactin), the ER (KDEL and Syntaxin 5) and the plasma membrane (Syntaxin 5). GalT was predominantly enriched in fractions containing Golgi membranes, but was additionally found in fractions reflecting the plasma membrane. Vertical bars to the left indicate the sedimentation profile: ER, endoplasmatic reticulum; PM, plasma membrane. Scale bars: 20 µm.
beta-1,3-galactosyltransferase (GalT) (Morin et al., 2001), enriched predominantly in Golgi membranes (Fig. 1D), exhibited a distribution similar to that of GFP-Drab6: it accumulated in the center of the oocyte at stage 8, where it colocalized with Drab6, and was later confined to the cortex (Fig. 1C). Importantly, the distribution of Lva and GalT was similar in both matHTubGFP-Drab6, ubiRFP-Drab6 and control oocytes (see Fig. S3 in the supplementary material). Given that Lva and GalT markers are not present in the Golgi cisternae that are evenly distributed throughout the oocyte, as documented by electron microscopy (EM) analysis (Herpers and Rabouille, 2004), they might be the hallmark of distinct functional Golgi units, with Drab6 being able to interact with both types of Golgi. Unlike Lva, the distribution of which was only mildly affected (Fig. 1C), GalT and acetyl-glucosamine-modified proteins [detected by the wheat germ agglutinin lectin (WGA)] expressed by Golgi structures were abnormally distributed in Drab6 mutants (Fig. 1C). Moreover, ultrastructural analysis by EM revealed that the ER was abnormally swollen in Drab6 mutant oocytes (Fig. 2A’ versus B’), and that the Golgi mini-stacks were markedly curved, with partially inflated cisternae (Fig. 2A’ versus B’).

These morphological effects led us to investigate the role of Drab6 in the secretory pathway. We monitored the polarized secretion of the TGFα-like growth factor Grk (Neuman-Silberberg and Schupbach, 1993). Grk secretion is restricted to the anterodorsal corner through a rapid transit from the ER towards the Golgi apparatus (Herpers and Rabouille, 2004). In GFP-Drab6-rescued egg chambers, Grk and Drab6 colocalized (Fig. 2C). In Drab6 mutant oocytes, grk mRNA localization was the same as in wild type (see Fig. S1 in the supplementary material). Grk protein, however, was slightly more abundant than in controls and an important fraction extended ventrally (Fig. 2E). Polarized secretion of Grk led to the formation of two dorsal appendages on the egg shell (Fig. 2D). In the absence of Drab6, mislocalized Grk induced ventralization (Fig. 2E, inset; 22% absent dorsal appendages, 28% fused, n=199), instead of a dorsalization (multiple dorsal appendages on the egg...
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plasmalemma, as shown in cell cortex, Drab6 controls the delivery of membrane to the plasma membrane. This phenotype is specific to Drab6 because it could be fully rescued by the GFP-Drab6 transgene (Fig. 2C).

Next, we analyzed the intracellular localization of Grk in the absence of Drab6. Grk accumulated frequently in large ring-like particles in the Drab6 mutant, but not in control oocytes (Fig. 2D,E). These Grk ‘rings’, similar to those of yolk granules (Bokel et al., 2006; Queenan et al., 1999), did not contain Lva (Fig. 2G), suggesting that Grk is not blocked in the Golgi. Grk actually accumulated in Drab6 mutants on vesicles stained by LysoTracker (Fig. 2H versus I), which labels either lysosomes or late endosomes containing yolk granules (Dermaut et al., 2005). Hence, two independent approaches suggest that Grk is not blocked in the Golgi, but is mislocalized to post-Golgi compartments, probably endosomes.

Interestingly, the secretory impairment was also confirmed by *Lycopersicon esculentum* tomato lectin (LE) detecting modified proteins in the Golgi. In the absence of Drab6, LE revealed abnormal vesicular structures in the oocyte and nurse cells that had failed to reach the cortex (see Fig. S2 in the supplementary material). EM analysis also demonstrated rupture of the plasma membrane between neighboring nurse cells (see Fig. S2 in the supplementary material). Finally, we observed that GFP-Drab6-rescued egg chambers exhibited an accumulative enrichment of Drab6 at the plasma membrane during oogenesis, which was particularly evident in nurse cells (see Fig. S2 in the supplementary material). This is consistent with the involvement of Drab6 in secretion towards the plasmalemma.

We have established the existence of three important and novel aspects of Drab6 function during oogenesis, as follow.

First, consistent with its localization in vertebrate cells, Drab6 is predominantly localized to the Golgi complex in *Drosophila*, but overlaps with Golgi markers that have distinct localizations, suggesting that Drab6 might associate with distinct functional Golgi units. Drab6 might also play a role in membrane exchange between Golgi and ER and in Golgi organization, according to our EM analysis, which is again consistent with known functions of mammalian Rab6 (Del Nery et al., 2006; Martinez et al., 1997; Queenan et al., 1999), did not contain Lva (Fig. 2G), suggesting that Grk is not blocked in the Golgi. Grk actually accumulated in Drab6 mutants on vesicles stained by LysoTracker (Fig. 2H versus I), which labels either lysosomes or late endosomes containing yolk granules (Dermaut et al., 2005). Hence, two independent approaches suggest that Grk is not blocked in the Golgi, but is mislocalized to post-Golgi compartments, probably endosomes.

Second, by controlling the migration of Golgi units towards the cell cortex, Drab6 controls the delivery of membrane to the plasmalemma, as shown in Drab6 mutants in which glycosylated proteins labeled by WGA and LE lectins accumulate in large vesicular structures. This pattern is similar to the mislocalization profile of Grk in the absence of Drab6.

Third, in the oocyte, Drab6 is required for the anterodorsal secretion of Grk, which leads to the differentiation of the follicle cells required for the morphogenesis of the dorsal appendages of the egg shell. In the absence of Drab6, we observed that Grk is mislocalized to late endosomal or lysosomal compartments, demonstrating that Drab6 also affects post-Golgi traffic. In vertebrates, one of the Rab6 isoforms (Rab6A') is also involved in endosome-to-Golgi transport (Del Nery et al., 2006; Utskarpen et al., 2006). Additionally, a role for Ypt6p (the only copy of Rab6 in the yeast *S. cerevisiae*) has also been documented as being involved in fusion of endosome-derived vesicles with the late Golgi (Siniossoglou and Pelham, 2001). It remains to be established whether Drab6 functions directly in the secretory pathway or if the effects observed in Drab6 mutants on post-Golgi trafficking are a consequence of defects in endosome-to-Golgi trafficking.

In order to identify potential Drab6-binding proteins, we performed a yeast two-hybrid screen (Formstecher et al., 2005) using as bait Drab6Q71L, a GTPase-deficient mutant. Sixty-two distinct truncated clones of BiCD, lacking parts of the amino-
terminus, interacted with Drab6Q71L (data not shown). The intersection of all identified fragments defined a minimal interacting domain, mapping to amino acids 699-772 in the coiled-coil motif H4 of BicD (Fig. 3A), shown for murine BicD to interact with the mammalian Rab6 (Matanis et al., 2002). In order to validate this interaction, we performed glutathione S-transferase (GST) pull-down assays, using lysates from wild-type ovaries. GST-Drab6 specifically retained BicD, as GST alone and GST-Rab1 did not bind BicD. Furthermore, preloading GST-Drab6 with the non-hydrolyzable GTP analog, GTP-γ-S, yielded an improved interaction with BicD (Fig. 3B). We conclude, therefore, that in vitro, BicD interacts through its carboxy-terminus preferentially with the active form of Drab6 (GTP-bound), as has been shown for mammalian Rab6 (Matanis et al., 2002; Short et al., 2002).

Time-lapse recording showed that in the oocyte and nurse cells, GFP-Drab6 and BicD-GFP (Pare and Suter, 2000) colocalize to multiple large aggregates with low dynamics (Fig. 3B). Further GFP-Drab6 accumulation in the center depended on the presence of BicD during stage 8, as observed in a BicDmutant background (see Swan and Suter, 1996) (Fig. 3C). Interestingly, in such BicDmutant oocytes, Grk was found in ring-like structures remote from the nucleus, as observed in Drab6 mutant oocytes (Fig. 3C).

Since BicD and Rab6 have been shown to be involved in MT-based transport, we checked whether Drab6-positive structures require MTs to move. Time-lapse microscopy revealed that large aggregates were less dynamic than the highly motile small particles. Colchicine MT depolymerization severely reduced the movement of Drab6 particles, which formed large clusters (Fig. 4B), indicating that Drab6 is actively transported along MTs. The MT motors Kinesin I [Kinesin heavy chain (Khc)] and Dynein have been shown to be involved in polarizing the Drosophila oocyte (Brendza et al., 2000; Brendza et al., 2002; Duncan and Warrior, 2002; Januschke et al., 2002). Inactivating the Dynein complex by the overexpression of Dynamitin (Januschke et al., 2002) prevented accumulation of Drab6 at the oocyte cortex (Fig. 4C), but did not significantly reduce Drab6 movements (Fig. 4C').

We observed that Drab6 and BicD interact in a yeast two-hybrid screen and in GST pull-down assays and colocalize in vivo. Moreover, there were indications that Drab6 requires BicD for correct subcellular localization, which suggests that Drab6 interacts with BicD in Drosophila as it does in mammals. Strikingly, we found that lack of each protein compromises Grk secretion in a very similar way. Overexpression of Dynamitin, to impair Dynein function, induces ectopic accumulation of Grk and ventralization of the egg shell (Januschke et al., 2002). Therefore, in Drosophila, BicD/Dynein and Drab6 are likely to be involved together in Grk secretion to the anterodorsal corner of the oocyte.

It is important to mention that colocalization of the two proteins was limited. Moreover, lack of BicD or Drab6 yields different phenotypes. BicD mutation affects oocyte determination and the position of the oocyte nucleus (Swan et al., 1999), but has no impact on MT organization in mid-oogenesis (Swan and Suter, 1996), which is not the case in the Drab6 mutant (see Fig. S1 in the supplementary material). A genetic interaction between BicD's co-factor Egalitarian and Kinesin I has already been demonstrated (Navarro et al., 2004), suggesting that Drab6 might interact with Dynine and Kinesin I via BicD.

Fig. 4. Drab6 is actively transported along microtubules. Frames taken from time-lapse recordings of GFP-Drab6-expressing Drosophila egg chambers. Untreated (A) GFP-Drab6-rescued egg chamber and (B) colchicine-treated egg chamber. (C) GFP-Drab6-expressing egg chamber overexpressing Dynamitin and (D) Khc7.288 germ line clone expressing GFP-Drab6. Below each panel, parameters of vesicle movement derived from the time-lapse recordings are indicated (A’–D’). Particle parameters were determined using ImageJ. Particles in oocytes and nurse cells were traced in a single optical plane in three different egg chambers for each: control, Khc clones and overexpression of Dynamitin. (A, B) Colchicine treatment abolished accumulation in the center as seen in controls (arrow) and particles seemed to form bigger clusters. (C) Overexpressing Dynamitin reduced accumulation of Drab6 at the cortex (arrow). (D) Stage 8 Khc7.288 oocyte expressing GFP-Drab6. Drab6 did not accumulate in the center and formed clusters close to the oocyte nucleus (asterisks). Scale bars: 20 μm.
Interestingly, we noticed that in the absence of Drab6, osk mRNA was not correctly localized in the oocyte (see Fig. S1 in the supplementary material) (42% dot, 31% diffuse, 10% undetectable, remainder wild type, n=75). 

gurken and bicoid mRNAs were, however, unaffected (see Fig. S1 in the supplementary material), and osk mRNA localization to the oocyte center is frequent when the MT network is not correctly polarized (St Johnston, 2005). In Drab6 mutant oocytes, the defective posterior localization of the MT plus-end marker Khc–β-Gal (Clark et al., 1997) indicates a defect in MT organization (see Fig. S1 in the supplementary material). Similar observations have been reported recently (Coutelis and Ephrussi, 2007).

Given that Drab6 is required for late Grk signaling at the anterodorsal corner of the oocyte, it might also be involved in early germ line to soma signaling mediated by Grk, which controls MT organization (Gonzalez-Reyes et al., 1995; Roth et al., 1995). We think that this is unlikely. In the absence of this signaling, posterior follicle cells differentiate into anterior follicle cells and, as a consequence, the posterior structure of the egg shell, the aeropyle, is substituted with an anterior structure, the micropyle (Gonzalez-Reyes et al., 1995; Roth et al., 1995). We always observed an aeropyle at the posterior of eggs derived from Drab6 mutant oocytes (see Fig. S2 in the supplementary material). Additionally, removing Drab6 from the posterior follicle cells did not affect oocyte polarity. Hence, Drab6 is possibly involved in MT organization at the posterior pole. Interestingly, Rab6 family interactors such as Rab6IP2/ELKS (Monier et al., 2002) are capable of interacting with CLASPs at the cortex of HeLa cells (Lansbergen et al., 2006), suggesting a link between Rab6 protein and MT organization at the cortex.

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Supplementary material
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